DNA damage and repair proteins in cellular response to sulfur mustard in Iranian veterans more than two decades after exposure

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Abstract

Delayed effects of sulfur mustard (SM) exposure on the levels of five important damage/repair proteins were investigated in 40 SM-exposed veterans of Iran-Iraq war and 35 unexposed controls. A major DNA damage biomarker protein – phosphorylated H2AX – along with four DNA repair proteins in cell response to the genome damage MRE11, NBS1, RAD51, and XPA were evaluated in blood lymphocytes from the veterans and controls using western blotting. Mean levels of XPA, MRE11, RAD51 and NBS1 were lower in SM-exposed patients and the decrease in NBS1 was significant. Even though the raised level of phosphor-H2AX in SM-poisoned group compared to

the controls was not significant it was consistent with DNA damage findings confirming the severity of damage to the DNA after exposure to SM. There were correlations between the values of RAD51 and NBS1 proteins as well as XPA and MRE11 proteins. More than two decades after exposure to SM, there is still evidences of DNA damage as well as impaired repair mechanisms in cells of exposed individuals. Such disorders in cellular level may contribute to long term health problems of the SM veterans.

Keywords: Sulfur mustard, veterans, damage/repair proteins, DNA damage, western blotting

Abbreviations

CHK1: checkpoint kinase 1 human protein

CHK2: checkpoint kinase 2 human protein

COPD: chronic obstructive pulmonary disease

DSB: double-strand breaks

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GSH: glutathione

γH2AX: phosphorylated H2AX

MRN: MRE11-RAD50-NBS1 complex

NAD^+: nicotinamide adenine dinucleotide

PARP-1: poly ADP-ribose polymerase-1

PBS: phosphate buffered saline

SM: sulfur mustard

1. Introduction

Genotoxicants which damage DNA directly or indirectly result in repair, mutation or cell death. Mutation happens while an alkylated base can mismatch repair when DNA is replicated. Genotoxicity tests such as in vitro mammalian cell gene mutation test, Ames test, comet assay, HPRT assay and mammalian erythrocyte micronucleus test are used to specify single and double strand breaks of DNA, cross links, point mutations and other sorts of DNA damage (1).

Chemical weapons were specifically designed to kill, injure, or incapacitate; among them vesicants such as sulfur mustard (SM) and lewisite cause blistering of skin and mucous membranes. SM is less lethal than nerve agents but poses more lasting injuries and disabilities in survived individuals (2, 3). SM caused over one million casualties in World War I and about 100,000 military and civilian Iranian victims during 1980-1988 Iran-Iraq war, many of them are still suffering from long-term exposure related illnesses (4, 5). Sulfur mustard is an oily liquid with garlic or mustard odor which has relatively low volatility and high environmental persistence. Many studies have demonstrated that SM has toxic effects on most body organs, particularly the eyes, skin, respiratory, hematologic and immunological systems (6-9). SM is an alkylating agent that is proved to remain cytotoxic and mutagenic effects due to having the potential to form the highly reactive episulfonium electrophile, which is capable of combining with nucleophilic sites of cells' macromolecules (10). This electrophile can inactivate sulfhydryl-containing enzymes and proteins, and also bring about products of interaction with DNA, RNA and lipid membranes. Consequently, SM acts as a genotoxic chemical and is a direct-acting carcinogen.

Although the reactions of SM with DNA and subsequent induction of cell death pathways have been documented, the exact mechanisms employed by cells to repair, is a question which is not fully understood. Acute cellular toxicity of SM is mediated mainly by the adduction (covalent addition) of SM with DNA; forming DNA monoadducts and inter/intra-strand cross-links (11). SM can be adducted to guanine base; preferentially attack site of DNA nucleotides and cross-links result from interaction with two guanine moieties due to bifunctional nature of SM which has two chloroethyl groups. Alkylating of O6 guanine is able to leave mutagenic lesions due to misreplication (12). SM also reacts with glutathione (GSH), the antioxidant content of cells. Depleted intracellular glutathione leads to loss of protection against oxidant stress; hence favors the production of endogenously produced oxygen species with subsequent peroxidation of membrane lipids (13). Normal cells attempt to recover from SM-induced DNA damage by

activating DNA repair enzymes such as nuclear enzyme poly ADP-ribose polymerase-1 (PARP-1). PARP-1 enzyme stimulates a DNA repair path through base excision repair pathway. However, high levels of cellular DNA damage result in cell death via apoptosis. The proposed mechanism is nicotinamide adenine dinucleotide (NAD^+) depletion that reduced-NAD^+ content in cells, results in ATP depletion. However, the molecular mechanism mediated by PARP-1 and ATP production during cell death, including necrosis and apoptosis, is not fully understood. DNA damage induction by SM induces PARP activation which in turn leads to cellular NAD^+ or ATP depletion. This decrease enhances synthesis and release of proteases that can provoke tissue injuries or cell death. The latter derives from the overstimulation of PARP-1 and induction of pro-inflammatory necrotic cell death, with high damage to neighboring cells and organ damages (14, 15). Even though cross-links form the minority of DNA damage than adducts (15% to 85%) they develop much more cytotoxicity. Genotoxicants can produce intra-strand cross-links (i.e., in the same strand) and inter-strand cross-links (i.e., between the two strands). These agents may cause excisions in cell's deoxyribonucleic acids. DNA excisions might happen as single- and double-strand breaks (DSB). Double strand breaks are serious lesions potentially lethal to cells unless accurately repaired. Cross-links may also induce double strand breaks. All these damage activate enzymes enrolled in DNA repair mechanisms such as PARP-1 (1, 16).

Several proteins are involved as a reaction on damage to the genome - like DNA double-strand breaks - to assist in repair of DNA breaks. The specific proteins activated after exposure to SM are of particular interest. After DNA double-strand breaks induced by ionizing radiation, H2AX is rapidly phosphorylated at the sites of DSB. It is now accepted that this phosphorylation takes place on Serine 139. Similarly, SM toxicity is able to cascade proteins that are involved in DNA damage signaling. The path initiates by the activation of protein kinases ATM, ATR, DNA-PK and some other as yet unknown kinases which phosphorylate H2AX. Phosphorylated H2AX (γH2AX) is critical for DNA degradation - forms nuclear foci at the sites of double-strand breaks by recruiting repair and checkpoint protein complexes. Moreover, ATM ad ATR phosphorylate Checkpoint kinase 1 human protein (CHK1), Checkpoint kinase 2 human protein (CHK2) and p53 tumor suppressor protein. CHK1 and CHK2 regulate cell functions and if necessary apoptosis (17, 18).

Nucleotide excision repair which is a specialized type of DNA repair is organized by XPA – a scaffolding protein via its interaction with other essential proteins (19). NBS1, also nibrin, is a protein associated with the repair of double strand breaks. NBS1with binding to H2AX plays a

key role in recognizing and rejoining double-strand breaks via activation of the ATM protein kinase (20). MRE11 is another DNA repair protein which is in complex with a member of the RAD family and NBS1 bringing about the nuclease activity. One of the known DSB repair pathways depends upon the recombinase function of RAD51 and the assistance of various other proteins (21, 22).

Since existing evidences suggest the role of SM in DNA damage and possible defects in DNA repair pathways in chronic health problems among exposed individuals, our study mainly focused on the cellular levels of specific DNA damage/repair proteins (γH2AX (Ser139), XPA, MRE11, NBS1, RAD51) in the mononuclear lymphocytes of exposed and unexposed populations.

2. Material and methods

This cross-sectional study was performed by selecting 40 male SM-exposed veterans and 35 suitable controls in Khorasan Razavi province, northeast Iran. Unexposed populations were considered as the control group who were family members or close relatives of the exposed individuals in a matched age range and geographical distribution. The veterans were all with confirmed history of battlefield exposure to SM between 1980-1988 according to the medical records and military service documents in the war veterans' foundation. Individuals with occupational exposure to chemicals, industrial fumes and possessing medical conditions known to be associated with health complications such as collagen vascular diseases, organ transplantation and radiation therapy were excluded from the study. Before sampling, the study was approved by the Research Ethics Committee of Mashhad University of Medical Sciences (MUMS) as well as the ethic committee of Janbazan Medical and Engineering Research Center (JMERC). It was conducted in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice.

Peripheral blood samples (20 mL) were obtained from brachial vein of each individual by venipuncture. Blood samples were collected in vacutainer tubes with anti-coagulant lithium-heparin additive for DNA damage/repair study. Immediately, lymphocytes were isolated from the whole blood using Amersham Ficoll-Paque PLUS lymphocyte separation medium (Lympho Prep.). Ficoll (15 mL) was added to each clean tube and blood samples were carefully layered over them. Samples were centrifuged for 20 min at 2800 rpm, 4ºC. The gradient-separated lymphocytes

were harvested gently from the interphase, washed twice with 10 mL PBS (phosphate buffered saline) (1:1), and centrifuged at 1500 rpm for 10 min.

Purified lymphocytes were counted in a Neobauer chamber using Trypan Blue solution 0.4% and viability of cells was more than 85%. Lymphocytes were then lysed by adding cell lysing buffer (Tris-EDTA buffer) and then boiled at 95°C for 5 minutes to denature the proteins. Proteins were aliquoted in 500 µL micro-tubes and kept at -70°C freezer prior to analytical testing by western blot in laboratories of Medical Toxicology Centre of Newcastle University, UK. Duplicate lymphocyte samples were prepared for each study subject.

2.1 Western blotting

Lysates were boiled for 5 minutes prior to sonication to shear the cellular DNA. Protein concentrations were determined using Coomassie Bradford Reagent and 20 µg of protein per well separated on 12% bis-tris gels before transfer to Hybond-C nitrocellulose membrane using an iBlot machine. Membranes were probed using standard protocols and evaluated according to the manufacturer's protocol for primary antibodies anti-XPA [Santa Cruz Biotech, Rabbit], anti-MRE11 [Novuss Biologicals, Rabbit], anti-Rad 51 [Santa Cruz Biotech, Rabbit], anti-NBS1 [Novuss Biologicals, Rabbit], anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) [Abcam, Mouse (housekeeper gene)] and anti-P-Histone H2AX [Cell Signaling Technology, Rabbit]. Membranes were then washed in TBST followed by incubation with the relevant HRP-conjugated secondary antibody. Membranes were washed thoroughly with TBST and visualized using chemiluminescence reagent. Images were captured and analyzed using a Syngene G: Box gel documentation system. Protein bands were quantified using GeneTools software (Syngene) and normalized to the levels of GAPDH. Controls supplied by the manufacturer were analyzed in parallel. Cell extracts of the TK6 lymphoblastoid cells were used as positive control in each gel. SeeBlue Plus2 was used in each gel as molecular weight marker.

3. Results and Discussion

3.1 Study populations

To delineate how SM-mediated DNA damage is related to the damage/repair proteins, 40 sulfur mustard exposed male veterans and 35 non exposed control individuals from Mashhad city were chosen as the study population. The control group was matched to the case group in terms of age, social background and geographical distribution. The mean±SD age of the subjects was 46.85±6.32 and 44.37±9.8 for exposed and unexposed groups, respectively. The difference between mean ages of two groups was not statistically significant (P = 0.16). Among the SM-exposed group, 14 (35%) were 18 years old or younger at the time of exposure. Mean of years passed after SM exposure was 24.3±0.57. Analysis of the clinical data revealed that the most common class of pathology among the exposed group was respiratory, followed by ocular and skin. Among exposed cases, 58.3% had mild lesions, 19.2% moderate and only 5.8 % had severe lesions in their lung, eye and skin. Rest of the cases (16.7%) had no significant clinical problems at least in one of the three mentioned organs. The most common complaints in the respiratory system examination were dyspnea (82.5%, N=33) and chronic cough (72.5%, N=29); in accordance with the previous studies that the lung is the most vulnerable organ to late damage from sulfur mustard in the human body presenting with a COPD pattern (23). In the dermatology examination, the most common complaints were itching (80%, N=32) and dry skin (57.5%, N=23). In the ophthalmology examination, the most common ocular problems were dry eye (77%, n=30) followed by visual loss (51.3%, N=20) and foreign body sensation (38.5%, N=15).

3.2 Western blotting results

The lung complication due to SM poisoning is now well defined as “Mustard Lung” and it is believed to be a unique phenomenon with possible destruction in the ability of cells in repairing mechanisms in response to DNA damage. Cellular DNA is subjected to numerous, constant attacks, both by endogenous and environmental agents. However, these potentially mutagenic effects are reduced by DNA repair proteins. Mutations in DNA repair genes following exposure to SM could explain increased levels of endogenous or exogenous DNA damage and a subsequent increase in DNA mutations. DNA damage signaling proteins selected for analysis in this study were selected based on prior in vitro studies that implicate their critical roles in the pathways related to SM toxicity (24). To investigate the possible effect of previous exposure to SM on the

DNA damage/repair system, the levels of five main proteins of these pathways were compared in exposed and unexposed groups (Tables 1 and 2) using the quantified data obtained by western blot study which was normalized by GAPDH.

Table 1: Western blotting levels of 5 proteins of interest in all exposed (N=40) and unexposed control (N=35) subjects

H2AX		XPA		NBS1		MRE11		RAD51	
Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed
0.965919	0.964834	0.134736	0.060487	0.428414	6.205019	2.289019	0.123108	0.490709	0.448672
0.011204	0.037127	0.004091	0.198082	0.191797	1.006664	0.017318	0.016432	0.124047	0.023452
0.048907	0.297875	0.001996	0.270703	0.091078	6.293877	0.007647	0.028917	0.047252	0.301533
0.012564	0.019681	0.001963	0.300427	0.007438	0.918019	0.001861	0.041366	0.086307	0.04649
0.75422	0.012457	0.248585	0.433532	2.486073	0.724341	1.293663	0.021059	0.645463	0.042737
0.139154	0.00667	0.004835	0.341011	0.897793	1.19703	0.00516	0.002266	1.019448	0.031253
0.958166	0.01271	0.000649	0.136773	0.08503	1.665445	0.005165	0.008402	0.053649	0.020763
0.135445	0.009093	0.245295	0.660389	1.144075	1.136129	0.089253	0.022917	0.051999	0.024855
0.111854	0.019809	0.273051	0.673766	1.181374	0.722263	1.042082	0.021015	0.048573	0.02387
3.014935	0.262559	0.251223	1.135848	7.173233	7.03724	0.638506	3.680781	2.267015	0.767044
1.705658	0.071401	0.241949	0.508512	0.566195	0.881065	1.235051	1.783048	1.552345	0.041299
0.402139	0.054885	0.291706	0.296967	0.51744	0.735407	0.016824	1.331879	0.19111	0.047521
0.286655	0.071146	0.034486	0.198923	0.970597	1.190987	0.148486	0.96549	0.218087	0.06138
0.251536	0.121233	0.073439	0.476278	3.146946	3.552906	1.543798	0.502684	0.247235	0.030657
0.092508	0.103677	0.38384	0.497695	3.369959	0.947934	1.070577	0.569461	0.087283	0.018211
0.042541	1.571363	0.173394	0.450431	0.816382	6.007659	1.829185	0.738516	0.052282	0.951975
0.073139	2.205168	0.123118	1.17794	0.493322	3.162786	0.26982	37.33863	0.004668	4.603016
0.072319	0.993452	0.151041	0.370767	0.537854	6.608862	0.935425	0.684282	0.028312	1.02429
0.035365	1.639867	0.162305	1.072745	0.502585	11.99342	1.378305	0.393734	0.026921	0.235251
0.065241	0.056599	0.171652	0.235216	0.648754	0.270701	1.696715	1.203276	0.042526	0.031276
0.072825	0.039013	0.182687	0.495062	1.063663	0.227262	1.834802	0.052298	0.00949	0.003099
0.054602	0.013216	0.212925	0.693571	0.255042	0.257575	1.525129	0.14257	0.006861	0.00373
0.056206	0.061449	0.252846	1.407991	0.592279	1.053367	1.761534	1.797403	0.273452	0.026083
0.073789	0.036069	0.479678	0.931931	0.194768	0.225831	1.087694	0.140741	0.003846	0.010745
0.012935	0.023918	0.685713	0.991857	0.769956	0.203495	2.928865	0.81163	0.015376	0.009226
0.088309	0.008755	0.878299	0.744264	0.730151	0.520176	2.94276	0.935505	0.019978	0.017277
0.016925	0.018106	0.251476	2.003294	0.277946	0.3395	1.658233	0.481413	0.004495	0.023902
0.014017	0.004526	0.509383	0.352101	0.267653	0.59458	0.387322	1.498044	0.004255	0.011132
0.194149	0.005363	0.410154	0.651905	1.239464	0.464349	6.41726	0.964879	0.073671	0.010798
0.929389	0.008292	0.820635	1.608929	0.318968	0.776094	0.341529	1.891071	0.00501	0.014469
0.030529	0.006726	0.16341	1.441658	0.145584	0.559308	0.333551	1.336752	0.010585	0.007389
0.058221	0.029995	1.118222	1.76456	0.6973	1.925869	1.240002	1.417003	0.027125	0.070684
0.019909	0.02143	0.616417	2.474897	0.802794	1.309776	0.550287	1.358001	0.002179	0.064638
0.507753	0.02792	0.500513	3.080412	0.34277	1.117027	0.330678	1.326174	0.001237	0.061449
1.928709	0.017823	0.709346	2.674346	2.813415	1.102697	2.10485	1.535992	1.104661	0.066631
1.939122	-	3.478735	-	0.98841	-	5.994186	-	0.23909	-
0.32255	-	2.52467	-	0.183499	-	3.188073	-	0.077556	-
0.072697	-	1.886583	-	0.70037	-	1.604273	-	0.012686	-
0.492602	-	1.746481	-	2.986503	-	5.02486	-	0.171977	-
0.049201	-	1.87094	-	0.674125	-	1.237691	-	0.085703	-

Table 2: Expression of DNA damage response proteins (Mean ±SEM) from blood lymphocytes of exposed (N=40) and control unexposed (N=35) subjects

Protein	Exposed	Unexposed	P value
H2AX	0.40±0.1	0.25±0.09	0.293
XPA	0.56±0.11	0.88±0.12	0.068
NBS1	1.03±0.2	2.08±0.45	0.041
MRE11	1.45±0.24	1.86±1.05	0.687
RAD51	0.23±0.07	0.26±0.13	0.860

As shown in Table 2, according to the results of protein immunoblotting study, the mean levels of XPA, MRE11, RAD51 and NBS1 which are DNA repair proteins were lower in the SM-exposed group than the non-exposed group. Figure 1 clarifies the comparison of aforementioned proteins values in case (SM-exposed) and control groups. However, NBS1 was the only protein which had significantly higher levels in non-exposed group. Although the mean level of γH2AX in exposed group was almost two folds of the unexposed group, but this difference was not statistically significant. Elevated level of phosphorylated H2AX (Ser139) - a DNA damage pathway protein - is consistent with results from concurrently performed analysis of the level of DNA damage measured by Comet assay (Figure 2) indicating the DNA damage after exposure to sulfur mustard (1). High DNA damage and H2AX expression after exposure SM might be due to reduced expression of repair proteins in the DNA damage cascade. Since that study revealed that there is no direct relationship between the severity of chronic phase clinical patterns of SM exposure and level of DNA damage, it suggests that SM exposure does not have a dose-dependent response on DNA damage more than two decades after the exposure.

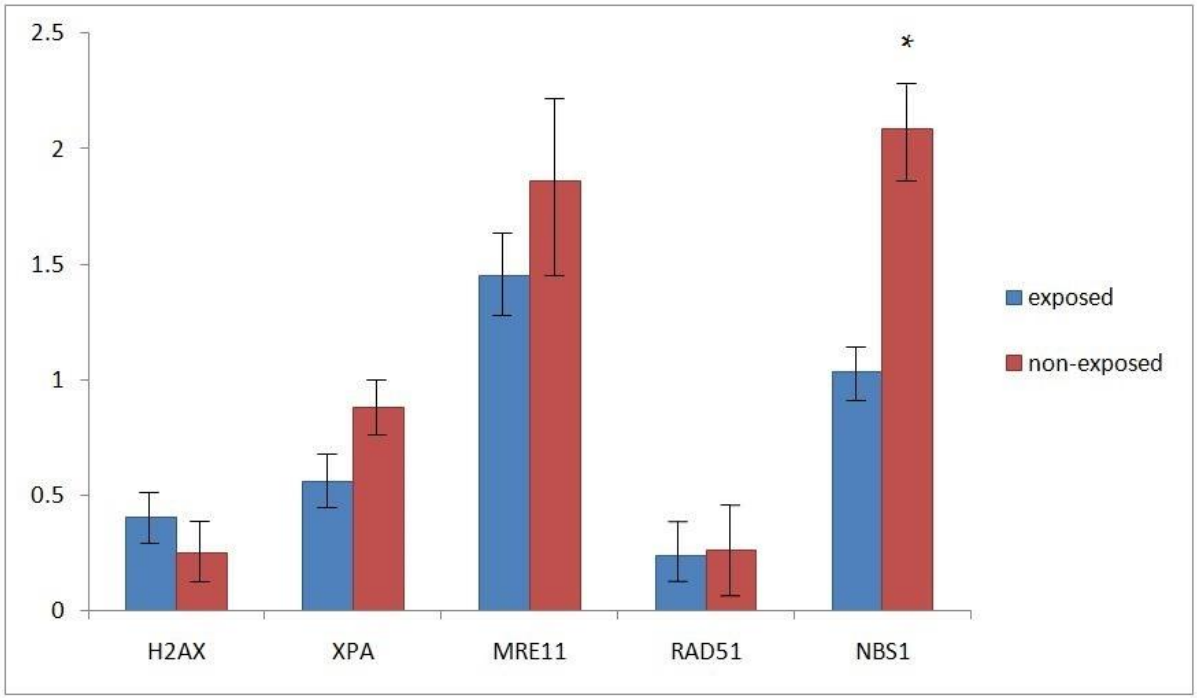

Figure 1: Comparison of the expression of DNA damage/repair response proteins in lymphocytes from SM-exposed subjects compared to unexposed individuals.
* The difference was statically significant between the two groups.

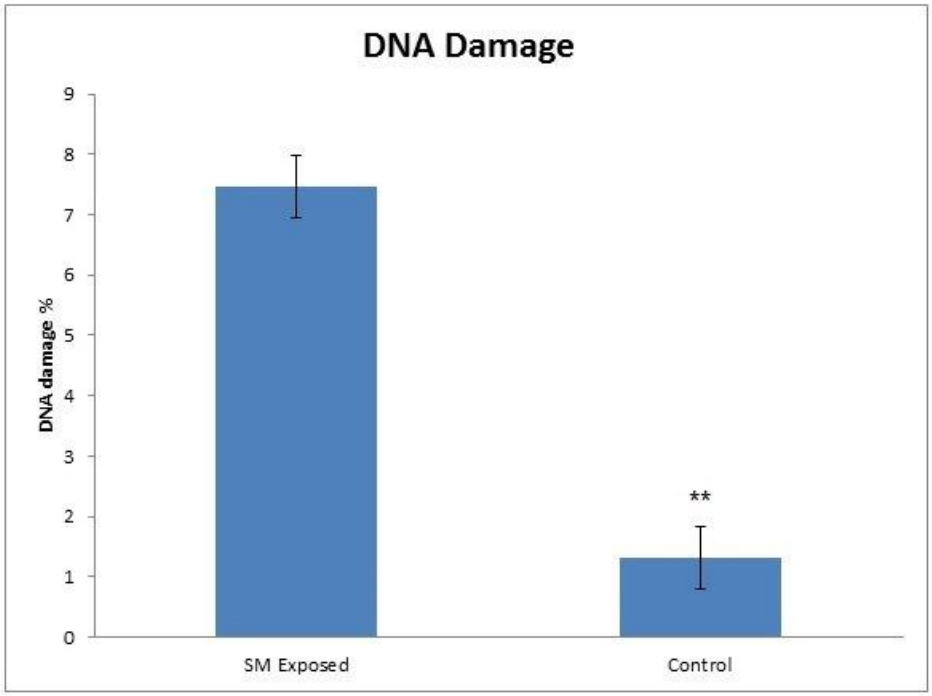

Figure 2: Human lymphocyte DNA damage in 25 exposed and control groups

** The difference was statically significant between the two groups.

In order to investigate possible relationship between the levels of DNA damage/repair proteins and clinical manifestation of exposure related illnesses, we considered the correlation between the levels of proteins and the severity of respiratory complications (Table 3). The examined veterans showed that based on their respiratory status they could be classified as mild (N=25), moderate (N=7), and severe (N=4) categories whereas 4 cases presented normal conditions.

Table 3: Mean ±SD response-to-exposure proteins in lymphocytes from SM-exposed veterans according to the severity of respiratory complications

Response-to-exposure proteins	**Respiratory difficulties**				
	Normal	Mild	Moderate	Severe	P value
H2AX	0.090±0.06	0.46±0.74	0.51±0.72	0.20±0.26	0.913
XPA	0.66±0.84	0.61±0.84	0.49±0.6	0.28±0.19	0.967
NBS1	0.75±0.37	1.04±1.46	1.47±1.49	0.60±0.24	0.981
MRE11	0.52±0.747	1.53±1.67	1.95±1.55	1.28±0.83	0.294
RAD51	0.27±0.5	0.25±0.53	0.28±0.4	0.03±0.03	0.545

The mean level of proteins of XPA and RAD51 is quite lower in SM-exposed veterans with normal respiratory status. H2AX damage protein showed gradual decrease trend that was lower than the veterans without obvious respiratory manifestations.

In a similar manner, possible relationship between the levels of DNA damage/repair proteins and ophthalmic problems was evaluated. The evaluation revealed that as the severity of the ophthalmic complications is increasing the levels of XPA DNA damage protein is decreasing; and the protein quantifications are lower in the veterans with normal ocular status. However, the difference is not significant ($P = 0.28$). XPA and NBS1 levels were higher in the veterans with normal ocular status. Evaluating the correlation between the protein values in lymphocytes from SM-exposed individuals revealed that there was significant correlation between the proteins RAD51 and NBS1 (P value < 0.001). The correlation evaluation also showed the same pattern for proteins MRE11 and XPA (P value < 0.001).

Difference between the levels of DNA damage response proteins was evaluated in veterans who were exposed to SM before or after the age of 18 years. DNA repair proteins XPA, NBS1 and MRE11 had higher mean levels in subjects who were over 18 years old at the time of incidence. It seems that the expected maintenance mechanisms of DNA repair was not performing as well at an earlier age. Age related evaluations continued regarding the levels of DNA damage response proteins' relation with years passed after mustard exposure. However, none of evaluated proteins had significant correlation with years passed after SM exposure.

We compared the mean level of proteins in smokers and non-smoker individuals. The differences between the number of smokers and non-smokers in both groups was not statistically significant (P = 0.15). Among the veterans, those who smoked cigarettes demonstrated more expressions of all the five studied response proteins (Table 4). The control populations followed the same pattern as well showing high damage/repair protein levels expression. This comparison is consistent with the previously stated findings on the mutagenicity of cigarettes.

Table 4: Correlation between the smoking habit and the levels of DNA damage response proteins among the SM-exposed group

	Smoking	N	Mean± SE	P Value
H2AX	yes	10	0.70±0.32	0.81
	no	30	0.30±0.08	
XPA	yes	10	0.62±0.32	0.86
	no	30	0.53±0.12	
NBS1	yes	10	1.52±0.65	0.22
	no	30	0.86±0.17	
MRE11	yes	10	2.28±0.70	0.14
	no	30	1.17±0.20	
RAD51	yes	10	0.41±0.21	0.10
	no	30	0.17±0.06	

As it is by now understood, formation of a complex of repair proteins is essential for the detection of DSB, recombination and initiation of DNA damage signaling. MRE11 and NBS1proteins as well as members of RAD family proteins form a complex (MRN) that acts to promote efficient DNA damage response via ATM signaling to maintain genome integrity (25, 26). This is consistent

with our results that there were correlations between the values of RAD51 and NBS1 proteins as well as XPA and MRE11 proteins. However, none had significant correlation with years passed after exposure.

In all samples of the case group possible correlation between the mean level of γH2AX and the DNA damage level based on the comet assay was studied (Figure 3). However, no significant correlation between these two values was observed (P = 0.39).

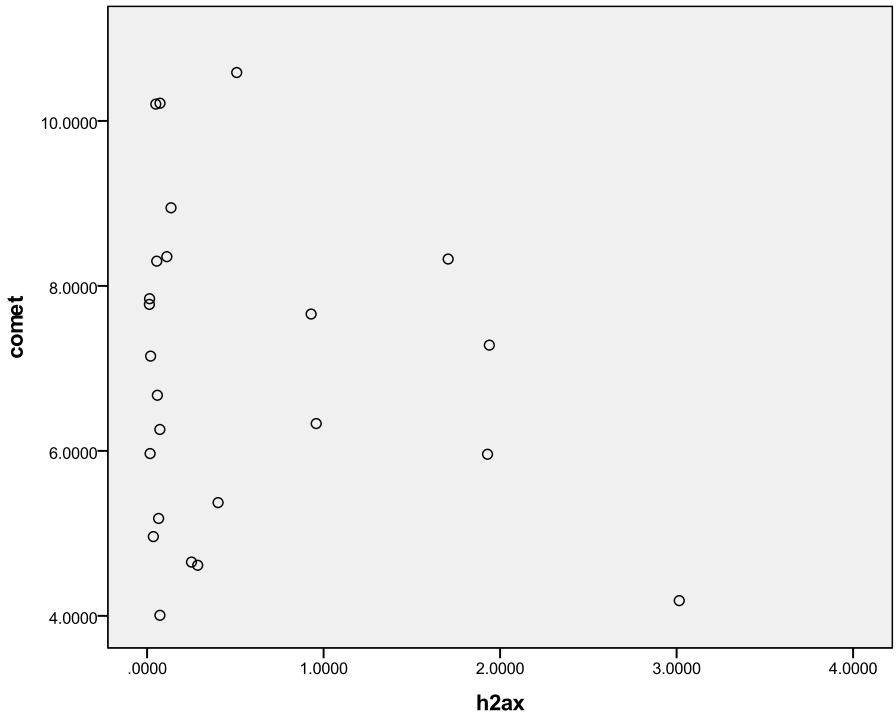

Figure 3: Correlation between γH2AX (Ser139) and the DNA damage level in SM-exposed population

4. Conclusions

We evaluated the levels of five important damage/repair proteins involved in cell response to genome damage in mononuclear lymphocytes of sulfur mustard poisoned veterans and unexposed controls using the western blotting technique. Mean levels of XPA, MRE11, RAD51 and NBS1 were lower in SM-exposed patients and the decrease in NBS1 was significant. Although the raised level of H2AX in SM-poisoned group was not significant in comparison to the controls but it was

in compliance with the comet assay findings from a concurrently performed study confirming the severity of damage to the veteran's DNA after exposure to sulfur mustard. Elevated phosphorylated H2AX level might be due to reduced expression of repair proteins in the DNA damage cascade. The same trend was observed in veterans who had normal respiratory functions. As it is by now understood, formation of administrative complex of repair proteins is essential for the detection of DSB, recombination and initiation of DNA damage signaling; which it is consistent with our results that there were correlations between the values of RAD51 and NBS1 proteins as well as XPA and MRE11 proteins. However, none had significant correlation with years passed after exposure.

To explain such important findings, we hypothesized that SM exposure causes mutations in DNA damage signaling proteins. This results in the impairment of the normal increase of phosphorylated H2AX in response to DNA damage. Perhaps the same mechanism explains the reduced quantity of some DNA repair proteins in SM-exposed individuals compared with healthy controls. We hypothesized that the disrupted DNA repair mechanisms could be one of the major reasons in the development of the chronic lesions observed in the exposed patients and its progressive nature.

More in depth studies of other DNA damage proteins and with a larger study sample may provide us with a better image of the long-term relationship between SM exposure and DNA damage signaling proteins as well as DNA damage. This would be useful in answering important questions regarding the mechanisms of acute and chronic SM toxicity and possible medical countermeasures to mitigate such toxicity.

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